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Resolution of (*RS*)-Proglumide Using Lipase from *Candida cylindraceae*

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Abstract—Proglumide is used in the treatment of neuropathic pain. It acts by inhibiting peptide cholecystokinin (CCK). Neural injury produces an elevation in plasma CCK. Proglumide has been also shown to augment the analgesic effect of sustained release morphine in neuropathic pain. Currently proglumide is administered as a racemic mixture. In the present study, an attempt is made to separate the racemic mixture of the drug using lipase obtained from *Candida cylindracea* by stereoselective esterification. Enzymatic stereoselective esterification was carried out in organic solvents. The resolution was studied using a chromatographic column with a chiral support and mass spectrometry. The reaction conditions for stereoselective esterification including amount of substrate, amount of enzyme, alcohol, solvent and temperature were optimised during the present investigation. Butanol and hexanol were found to be suitable for formation of *S* and *R* esters, respectively. Hexane was the best solvent for esterification and the optimum temperature was found to be 30 °C.

Introduction

Since the late 1980's, there has been an increasing trend to produce and market chiral drugs composed of single active enantiomers rather than as racemes which contain both the active and the inactive isomers.[1] Due to this evolution, there is an absolute need to produce new drugs with high optical purity and to enantioenrich old drugs so that they show a high optical purity.[2]

Lipases are widely used in racemic resolutions because of their stereospecific action. Many classes of lipases have been used for the preparation of pure enantiomers. Some well known examples of racemic resolution in the field of pharmaceuticals include production of *S*-ibuprofen and its derivatives, production of *S*-naproxen and its derivatives and production of *S*-captopril and the corresponding family of drugs.[3,4]

Proglumide is a pharmacological agent used in the management of neuropathic pain.[5] Neuropathic pain is associated with elevated levels of cholecystokinin (CCK).[6] Proglumide is a CCK antagonist and it augments the analgesic effect of sustained release morphine in the management of neuropathic pain.[7] Proglumide is chemically *N*-benzoyl-*N*′,*N*′-dipropyl-*RS*-isoglutamine (Fig. 1). Newer synthetic derivatives of proglumide have been synthesised in which the pharmacological activity resides in the *R*-isomer.[8]

Enzyme catalysed reactions have been carried out in aqueous and solventless media. The water content requirements for biocatalyst activity may vary widely as the solvent is changed. A much higher concentration is needed in polar solvent to give same availability in the environment of the active enzyme molecules. The water activity required for good catalytic activities differ for different lipases. Some lipases retain high activity even after drying over molecular sieve (lipase from *Rhizomucor miehei* and *Candida antartica*).[9,10]

A biocatalytic reaction in a nonaqueous solvent will limit the hydration of the enzyme and result in the production of the *S* isomer. In this paper we present the esterification of *N*-benzoyl-*N*′,*N*′-dipropyl-*RS*-isoglutamine for the production of the *N*-benzoyl-*N*′,*N*′-dipropyl-*S* (+)-isoglutamine and *N*-benzoyl-*N*′,*N*′-dipropyl-*R* (+)-isoglutamine using a lipase obtained

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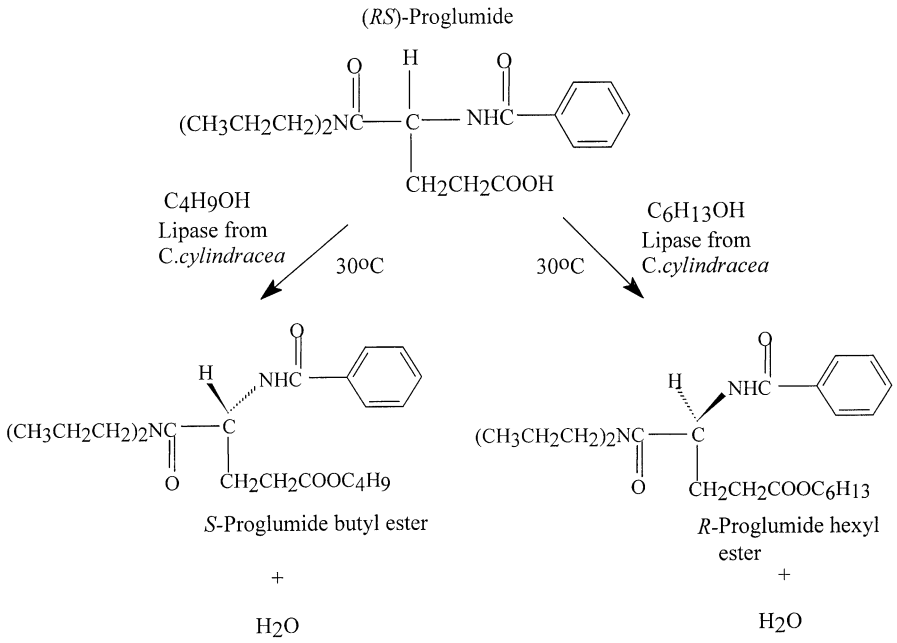

Figure 1. Chemical structure of proglumide and the esterification reactions catalysed by lipase from *Candida cylindracea*.

from *Candida cylindracea* by the process of stereoselective esterification. Conditions for racemic resolution including the amount of enzyme, amount of substrate, effect of alcohols, the effect of various solvents on esterification, the presence of water, and temperature will be discussed in detail.

Results and Discussion

Substrate concentration

Reactions were carried out at different concentrations (10–50 mg/mL with a step increase of 2.5 mg) of the substrate keeping the enzyme concentration constant (10 mg/mL). Butanol was used in the reaction mixture for obtaining (*S*)-ester and hexanol in the reaction mixture for obtaining (*R*)-ester. Hexane was used as the solvent. The substrate was dissolved in 1 mL of corresponding alcohol and 1 mL of solvent. There was a linear increase in the enzyme activity from 10 mg/mL of the substrate to 20 mg/mL. From 20 to 35 mg/mL, the enzyme activity remained constant. Substrate concentrations greater than 35 mg/mL caused a decrease in the resolution probably due to the inhibition of enzyme by substrate.

Amount of enzyme

These experiments were performed by keeping the substrate concentration constant but varying the amount of enzyme. The substrate concentration was fixed at 20 mg/mL and the enzyme concentration varied from 10 to 100 mg/mL. A gradual decrease in the reaction time was observed when the enzyme concentration was increased from 10 mg/mL to 50 mg/mL. An increase in the reaction time was observed after 50 mg/mL of enzyme concentration probably due to the fact that less amount of substrate is available to the enzyme at its active site for resolution.

Table 1. Esterification of (*RS*)-proglumide with different alcohols catalysed by lipase from *Candida cylindracea* in hexane at 30 °C, molar conversion and ee calculated after 33 h

Alcohol	Molar conversion (%)	ee[a] (%)	E[b]
Methanol	25	(*S*) 30	25
Ethanol	33	(*S*) 45	34
Propanol	39	(*S*) 60	58
Butanol	52	(*S*) 97	75
Pentanol	39	(*S*) 51	14
Hexanol	50	(*R*) 93	94
Heptanol	50	(*R*) 84	30
Octanol	43	(*R*) 71	70

[a]Enantiomeric excess of the ester.
[b]Enantiomeric differentiation of the ester.

Alcohol-substrate

Table 1 shows the effect of the carbon chain on the esterification of *R* and *S* isomers of proglumide. The reactions were carried out using hexane as the solvent. In primary alcohols at 30 °C, good enantiomeric excess of the *S*-ester was obtained. Butanol was found the best alcohol giving an enantiomeric excess of 97% for the *S*-ester. Use of higher alcohols resulted in a decrease in the resolution of the *S* ester. In case of *R*-ester, *n*-hexanol was found the best alcohol for esterification at 30 °C. The esterification of isomers in a racemic mixture of proglumide is dependent on the length of the alcohol chain. These studies indicate the esterification of *S* isomer is good at lower chain alcohols with chain length up to butanol. Increasing the carbon chain in alcohols preferentially prefer the formation of the *R*-ester. No esterification was observed with alcohols above nonanol. The enantioselective behaviour of lipase based on the nature of alcohol has been explained in detail in literature.[11] The molecular basis of enantioselectivity has been studied in detail.[12] The polarity of the substrate and the polarity of the reaction medium are believed to

determine the enantioselectivity of the enzyme. They are the two factors responsible for the conformational changes, which a lipase molecule can undergo in a biocatalytic reaction.[13]

Substrate-solvent

Enzyme activity depends upon the polarity of the solvent.[14] Solvents with different values on the polarity scale were examined. The reactions were performed at 30 °C or at temperatures lower than boiling points for solvents having lower boiling points. The enantioselectivity increased with increase in logP values with the exception of octane. For reaction in butanol using hexane as the solvent, enantioselectivity of 75 was obtained. With reaction in hexanol high *E* ratio of 94 was obtained using hexane as solvent. The least enantioselectivity for both the alcohols was obtained with diethyl ether (Table 2).

Effect of temperature on the production of *R*- and *S*-esters

Temperature increases the rate of a reaction and at higher temperatures, the reaction might stop due to deactivation of the enzyme. Resolution of the *S* ester was observed at temperatures ranging from 30 to 50 °C in hexane. The optimum temperature for esterification of both the esters was found to be 30 °C. Using butanol, an ee value of 97% was obtained at 30 °C. Other alcohols gave lower ee values for the *S*-esters. Below 30 °C, the *R*-ester was formed. An enantiomeric excess of 93% was obtained for the *R*-ester using *n*-hexanol at 30 °C. Results of the deactivation studies indicate that partial deactivation of the enzymes occurred above 50 °C with most of the alcohols. The experiments were stopped after 50 °C as deactivation affects the process of resolution. Results of effect of temperature are given in Table 3.

Effect of initial water content on the production of *R*- and *S*-esters

Water concentration was studied from 0.1 to 5%. At low concentrations of water (0.1–1%), the formation of the *S*-ester is greatly enhanced. Increasing the concentration of water from 1 to 5% resulted in the production of the *R*-ester. Highest ee value for *S*-ester (93%) was obtained at 0.1%, v/v of water using *n*-butanol and for the *R* ester, large ee value of 91% was obtained using *n*-hexanol (3%, v/v of water) (Table 4). The nature of solvents also plays a role in the stereospecificity of the enzyme. Hydrophilic solvents tend to remove water from the enzyme distorting its conformation, whereas hydrophobic solvents preserve the water layer forming a protective cover thereby hindering lipase activity.[15] The fact that esterification of *S* proglumide increases when the enzyme is less hydrated is interesting. Low hydration of the enzyme is believed to decrease the flexibility of the enzymes, impeding their catalytic activity. This is because of interactions between polar residues, which are no longer screened by bound water molecules. This phenomenon has been observed in chymotrypsin suspended in anhydrous hexane. The increased rigidity of trypsin is accompanied by a reorientation of side chains. This attributes to the production of *S* ester.[16]

Table 2. Esterification of (*RS*)-proglumide with butanol and hexanol catalysed by lipase from *Candida cylindracea* at 30 °C in different solvents, molar conversion and ee calculated after 33 h

Solvent	Butanol			Hexanol		
	Molar conversion (%)	ee[a] (%)	*E*[b]	Molarconversion (%)	ee[a] (%)	*E*[b]
Diethyl ether	30	35	14	20	41	5
Ethyl acetate	31	40	25	35	48	28
Benzene	35	48	27	37	54	40
Toluene	37	53	33	42	67	52
Pentane	38	57	49	42	69	85
Hexane	52	97	75	50	93	94
Octane	43	70	56	56	89	13

[a]Enantiomeric excess of the ester.
[b]Enantiomeric differentiation of the ester.

Table 3. Effect of temperature on production of *R* and *S* esters of proglumide by lipase from *Candida cylindracea*

	Temperature (°C)							Lipase activity (LU/mg) at corresponding temperature (°C)						
	20	25	30	35	40	45	50	20	25	30	35	40	45	50
	Enantiomeric excess (ee%)													
Methanol	(*R*) 33	(*R*) 47	(*S*) 30	(*S*) 61	(*S*) 54	(*S*) 39	(*S*) 27	645	681	710	721	633	612	533
Ethanol	(*R*) 27	(*R*) 56	(*S*) 45	(*S*) 53	(*S*) 49	(*S*) 32	(*S*) 23	645	679	710	720	633	612	533
Propanol	(*R*) 31	(*R*) 50	(*S*) 60	(*S*) 60	(*S*) 55	(*S*) 39	(*S*) 28	633	684	713	719	645	603	512
Butanol	(*R*) 41	(*R*) 67	(*S*) 97	(*S*) 93	(*S*) 87	(*S*) 72	(*S*) 65	631	670	709	715	683	624	537
Pentanol	(*R*) 33	(*R*) 39	(*S*) 51	(*S*) 51	(*S*) 49	(*S*) 38	(*S*) 31	641	681	712	720	632	602	523
Hexanol	(*R*) 37	(*R*) 35	(*R*) 93	(*R*) 44	(*R*) 49	(*R*) 31	(*R*) 33	645	673	705	715	630	623	612
Heptanol	(*R*) 31	(*R*) 35	(*R*) 84	(*R*) 42	(*R*) 35	(*R*) 27	(*R*) 33	633	675	712	718	641	593	512
Octanol	(*R*) 35	(*R*) 43	(*R*) 71	(*R*) 37	(*R*) 30	(*R*) 22	(*R*) 33	615	639	693	712	702	655	513

(*R*) indicates *R* ester; (*S*) indicates *S* ester; ee indicates enantiomeric excess of the ester.

Table 4. Esterification of (*RS*)-proglumide at different initial water concentration with ethanol and octanol catalysed by lipase from *Candida cylindracea* in hexane, ee (of the ester) calculated after 40 h along with molar conversion values

Water (% v/v)	Butanol		Hexanol	
0.1	93*S*	50	68*R*	60
0.3	90*S*	49	75*R*	50
0.5	85*S*	53	81*R*	48
1	70*S*	48	85*R*	54
3	55*S*	45	91*R*	65
5	30*S*	40	80*R*	50

Stability of lipase in bio-organic reactions

Enzyme stability is an important aspect in bioorganic reactions. The overall recovery of the enzyme is vital as the recovered enzyme can be reused for further reactions after a study of its activity.[17] Lipase from *C. cylindracea* was found reasonably stable in esterification of proglumide. After six repeated batch applications a decrease of 15% in the overall enzyme activity was observed. Higher alcohols namely hexanol heptanol and octanol, caused greater deactivation of the enzyme when compared to lower alcohols such as methanol, ethanol, propanol, butanol and pentanol. At temperatures lower than 35 °C and a moisture content less than 2% (v/v), 95–98% (w/v) of the total enzyme used could be recovered with a 1–3% loss in the total activity. With higher moisture content and temperature [above 2% (v/v) and 40 °C], the total protein recovered decreased to 80% (w/v) of the total enzyme used and the total activity decreased to 90% of the initial activity. This loss in the enzyme activity could be due to hydrolysis of the enzyme due to the increased moisture content and deactivation of the enzyme protein because of the rise in temperature of the reaction.

Conclusions

The optimum enzyme and substrate concentrations were found to be 20 and 50 mg/mL, respectively. Butanol was found to be the preferred alcohol for the formation of (*S*)-ester and hexanol for (*R*)-ester. Hexane was found to be the best solvent and and the optimum reaction temperature was found to be 30 °C. At lower concentrations of water (0.1–1% v/v), the formation of *S*-ester is greatly enhanced and higher water concentration (3% v/v) led to the formation of *R*-ester.

Experimental

Materials

Lipase from *C. cylindracea* (Northern Regional Research Laboratory, USA, Y-17506) was obtained from fermentative production in our laboratory. A partially purified and freeze dried enzyme was used for the present investigation. (±)-progluimide. All alcohols and solvents were obtained from Sigma Chemical Company, USA.

Reactions

The reactions for determining the substrate concentration and enzyme concentration were carried out in glass tubes incubated in a temperature controlled water bath. All other reactions were carried out in 100-mL conical flask with ground glass tops. The flasks were incubated at different temperatures with the help of a temperature-controlled incubator. Reactions were performed using 5 g/L of the benzoyl-*N*′,*N*′-dipropyl-*RS*-isoglutamine and 25 mg of enzyme (710 units/mg) in 25 mL of the solvent containing 15 mL of alcohol. The reactions were started by dropping the enzyme into the flasks containing the substrates in the respective alcohols. Samples were taken at every 4 h time intervals and analysed by using a chiral column. After esterification, the enzyme was removed by filtering the reaction mixture using a 0.25 μm filter and washing the enzyme with methanol. The enzyme was subjected to repeated batch experiments. The enzyme stability was also determined by studying its activity.

Analytical techniques

Lipase activity was estimated using substrate emulsion method using olive oil as the substrate. One lipase unit is defined as one microequivalent of fatty acid released per min by the enzyme under standard reaction conditions.[17] Protein was estimated by the method of Bradford.[18] The racemic resolution was studied by HPLC (Thermospec Inc., USA) using a chiral AGP column (Chromtech, Sweden) with 5% 2-propanol in 10 mM sodium phosphate buffer as the mobile phase at 225 nm. The formation of the *R*- and *S*-esters was further characterised by electron spray ionisation mass spectrometry using a Finnigan LCQ mass spectrometer.

Calculation of enantiomeric excess and enantioselectivity

The enantiomeric excess, ee, at a given time and the enantiomeric ratio *E* were calculated according to the method of Gandolfi et al. and Damle et al.[19,20] The ee and *E* values mentioned are an average of triplicate values with a deviation of 5%. The outcome of reactions was mentioned in terms of enantiomeric excess or enantioselectivity.

Study of effect of initial water concentration

The effect of initial water concentration on the esterification of the *R* and *S* isomer were studied by using different concentrations of water in the reaction mixture. The water concentration in the reaction mixture was increased from 0.1 to 5%. The reaction was carried out in glass stoppered conical flasks agitated at 250 rpm for 36 h and samples were taken at 4 h intervals for chiral AGP analysis and mass spectrometry.

Study of effect of reaction temperature on the production of *R*- and *S*-isomers

After screening for a suitable solvent, the reaction temperature required for optimal esterification of *R* and *S*

isomers was done in a temperature-controlled incubator. The reaction temperature was studied form 20 to 50 °C. The enzyme activity was also studied simultaneously to observe deactivation of the enzyme. The enzymes were placed in the corresponding organic alcohol-solvent mixtures containing the pure substrate. The reaction temperature was maintained as stated above with a step-change in temperature of 5 °C. After every 4 h, samples were taken; the lipase activity was determined by the substrate emulsion method and the resolution was studied by chiral HPLC analysis.

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